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SNARE Complex Oligomerization by Synaphin/Complexin Is Essential for Synaptic Vesicle Exocytosis

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Summary

Synaphin/complexin is a cytosolic protein that preferentially binds to syntaxin within the SNARE complex. We find that synaphin promotes SNAREs to form precomplexes that oligomerize into higher order structures. A peptide from the central, syntaxin binding domain of synaphin competitively inhibits these two proteins from interacting and prevents SNARE complexes from oligomerizing. Injection of this peptide into squid giant presynaptic terminals inhibited neurotransmitter release at a late prefusion step of synaptic vesicle exocytosis. We propose that oligomerization of SNARE complexes into a higher order structure creates a SNARE scaffold for efficient, regulated fusion of synaptic vesicles.

Introduction

In nerve terminals, the rapid, calcium-regulated exocytosis of neurotransmitters is mediated by a cascade of interactions among membrane and soluble proteins (Rothman, 1994; Südhof, 1995; Augustine et al., 1999; Lin and Scheller, 2000). The fusion of synaptic vesicles and plasma membranes that underlies neurotransmitter release requires interactions between the proteins and lipids of these two membranes. Among the protein constituents, SNARE (*SNA*P *re*ceptor) proteins are of central importance for these interactions. The v-SNARE, synaptobrevin 2 (also known as VAMP2), resides on synaptic vesicles and binds to t-SNAREs, syntaxin and *syn*aptosome-*a*ssociated *p*rotein of 25 kDa (SNAP-25), on the plasma membrane. Many lines of evidence indicate that these three proteins act at a step that follows docking of synaptic vesicles at the plasma membrane (Hunt et al., 1994; Broadie et al., 1995; Sweeney et al., 1995). Reconstituting SNARE proteins into liposomes demonstrates that these proteins can serve as a minimal molecular machinery to fuse membranes (Weber et al., 1998), though it is presently unclear whether SNAREs directly mediate membrane fusion or act at a step that immediately precedes fusion (Coorssen et al., 1998; Ungermann et al., 1998).

In vitro, SNARE proteins bind to each other to form binary and heterotrimeric complexes (Hayashi et al., 1994; Pevsner et al., 1994). The ternary SNARE complex is highly stable and resists denaturation by detergents such as SDS (Hayashi et al., 1994). It consists of a parallel four-helix bundle containing one coiled-coil domain from syntaxin, another from synaptobrevin 2, and two from SNAP-25 (Sutton et al., 1998). Dissociation of the complex appears to require NSF, an ATPase that attaches to the SNARE complex via α-SNAP (Söllner et al., 1993a, 1993b). These interactions among the SNARE proteins seem important for neurotransmitter release, because neurotransmitter release and other forms of exocytosis are affected by conditions that alter SNARE interactions (DeBello et al., 1995; O'Connor et al., 1997; Littleton et al., 1998; Schweizer et al., 1998; Chen et al., 1999; Xu et al., 1999).

Although SNARE complexes are important for neurotransmitter release, it is not yet clear how their assembly is regulated in vivo. A number of cytosolic proteins have been found to bind to SNAREs in vitro and it is possible that these proteins regulate SNARE complex assembly. One potential regulator of SNARE assembly is synaphin (also called complexin; Ishizuka et al., 1995; McMahon et al., 1995; Takahashi et al., 1995). This soluble protein binds to syntaxin, in particular when syntaxin is complexed with the other SNAREs (McMahon et al., 1995; Pabst et al., 2000). Further, binding of synaphin prevents α-SNAP from binding to the ternary SNARE complex (McMahon et al., 1995), which in turn inhibits dissociation of this complex by NSF. These in vitro results suggest that synaphin regulates SNARE complex dynamics and thereby plays a central role in synaptic transmission.

Here, we have used the squid giant synapse to study the function of synaphin. We report that synaphin facilitates the association of SNAREs into an intermediate complex that can oligomerize into higher order structures and that these SNARE oligomers are required for the fusion of docked synaptic vesicles. We propose that the function of synaphin is to organize *trans*-SNARE complexes into oligomeric scaffolding structures that facilitate interactions between the synaptic vesicle and plasma membranes required for rapid membrane fusion.

Results

Identification and Characterization of a Squid Synaphin Ortholog

To study the role of synaphin at the squid giant synapse, we first identified its squid counterpart. Squid optic lobe synaptosomes contained a protein of 21 kDa apparent

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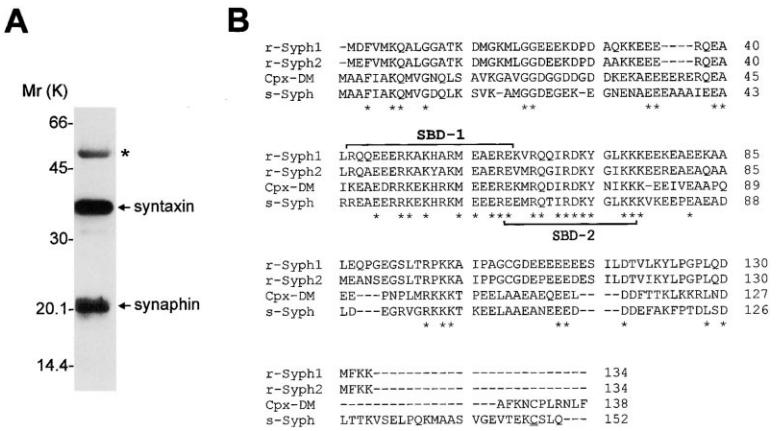

Figure 1. Identification of Squid Synaphin

(A) Coimmunoprecipitation of synaphin and syntaxin from optic lobe synaptosomes by an anti-syntaxin antibody. Western blotting with anti-syntaxin and anti-synaphin antibodies revealed the presence of both proteins; asterisk indicates IgG heavy chain.

(B) Alignment of amino acid sequences of squid and rat synaphins (Syph), as well as *Drosophila* complexin (Cpx-DM). Identical amino acids are marked by asterisks, while brackets indicate SBD-1 and SBD-2 peptides, and the double underlined cysteine a predicted prenylation site. Dashes correspond to gaps introduced to maximize alignment.

molecular mass that was recognized by a monoclonal antibody directed against rat synaphin 1. This protein was coimmunoprecipitated with syntaxin from detergent extracts of squid optic lobe synaptosomes, suggesting an interaction between these two proteins in vivo (Figure 1A). This 21 kDa protein was abundant in the cytosol of optic lobe and was slightly larger in size than rat synaphins, which have an apparent molecular mass of 19 kDa (Ishizuka et al., 1995; McMahon et al., 1995; Takahashi et al., 1995).

Partial sequences of proteolytic peptide fragments derived from affinity-purified squid protein were used to generate a PCR fragment for screening a squid optic lobe cDNA library. More than 30 positive cDNA clones were sequenced and all encoded the same protein, suggesting that there is a single form of synaphin in squid (Figure 1B). The amino acid sequence of squid synaphin is 47% identical to rat synaphins and 57% identical to the *Drosophila* homolog (Pabst et al., 2000). Sequence identity is particularly high in the central portion (residues 42–78), with identity values of 70%–73% to the rat, and 81% to the *Drosophila*, proteins.

To study the binding properties of squid synaphin, we incubated recombinant synaphin with increasing concentrations of the cytoplasmic domain of rat syntaxin, in the presence or absence of synaptobrevin 2 and/or SNAP-25. Though syntaxin alone bound only weakly to synaphin (Figure 2A, top row), the presence of other SNAREs enhanced this interaction. Specifically, binding was increased by adding either synaptobrevin 2 or, more robustly, SNAP-25 (Figure 2A, middle rows), and optimal binding was observed with all SNAREs present (Figure 2A, bottom row). Thus, s-synaphin, like its mammalian counterparts, preferentially binds to syntaxin in the ternary SNARE complex. Further, mammalian α-SNAP caused a concentration-dependent inhibition of s-synaphin binding to syntaxin (Figure 2B), as described for the mammalian proteins. From these data, we conclude that s-synaphin shares with its mammalian orthologs both a high degree of sequence similarity and comparable binding properties.

The Syntaxin Binding Region of Synaphin

Because binding to syntaxin is likely to be central to the function of synaphin, we mapped its syntaxin binding region. Three truncated constructs encompassing either the NH_2-terminal region (residues 1–51), the central region (residues 52–102), or the COOH-terminal region (residues 103–152) were incubated with syntaxin-containing detergent extracts of squid synaptosomes. While the central region of s-synaphin bound to syntaxin, no binding was detected to the NH_2- or COOH-terminal

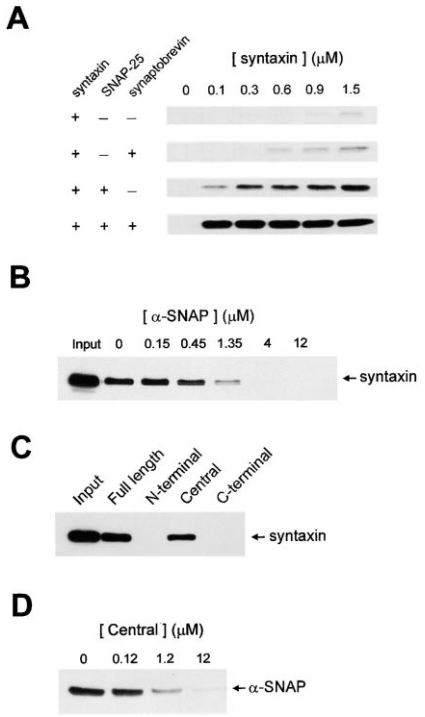

Figure 2. Binding Properties of Squid Synaphin

(A) Binding of GST-s-synaphin to recombinant r-syntaxin in the presence or absence of r-SNAP-25 (1 μM) and/or r-synaptobrevin 2 (2 μM), or both. Bound syntaxin was analyzed by Western blotting with an anti-syntaxin antibody.

(B) α-SNAP competitively inhibits binding of GST-s-synaphin to syntaxin from squid synaptosomes. Bound syntaxin was analyzed by Western blotting.

(C) Differential binding of recombinant s-synaphin constructs to syntaxin.

(D) Central region of synaphin competes with α-SNAP for binding to syntaxin. Bound α-SNAP was coprecipitated with immobilized anti-syntaxin antibodies and probed with anti-α/β-SNAP antibodies.

constructs (Figure 2C). Thus, the central region of synaphin is responsible for binding to syntaxin (see also Pabst et al., 2000). Like full-length synaphin (Figure 2B), the central fragment of s-synaphin inhibited α-SNAP binding to syntaxin (Figure 2D). This indicates that α-SNAP and synaphin compete for overlapping binding domains on syntaxin.

To study the functional importance of the synaphin–syntaxin interaction, we searched for a reagent that inhibits synaphin binding to syntaxin. The H3 domain of syntaxin, which is the binding site for synaphin (Pabst et al., 2000), was not appropriate for this purpose because many other proteins also bind within this region (Kee et al., 1995; O'Connor et al., 1997). Likewise, the central fragment of synaphin, which contains the syntaxin binding site, could not be used because it also prevents the interaction of α-SNAP with syntaxin. We, therefore, examined the actions of shorter peptides from the syntaxin binding domain of s-synaphin—specifically within the region of high evolutionary conservation (residues 42–78)—on the interactions of synaphin, syntaxin, and α-SNAP. Of these *S*yntaxin *B*inding *D*omain peptides (Figure 1B), only SBD-2 inhibited the binding of squid syntaxin to s-synaphin (Figure 3A). This inhibition was concentration dependent and half maximal at 0.6 mM (Figure 3B). Very similar results were obtained using r-SBD-2, from the same region of rat synaphin 1 (data not shown). Blockade of synaphin binding to syntaxin was sequence specific because neither scrambled SBD-2 peptides nor s-SBD-1, from an adjacent region of squid synaphin, interfered with binding of synaphin to syntaxin (Figures 3A and 3B and data not shown). Notably, neither s-SBD-2 nor r-SBD-2 prevented syntaxin from binding to α-SNAP (Figure 3C). Further, these peptides did not affect the interaction of syntaxin with its other binding partners, such as synaptotagmin, synaptobrevin 2, or SNAP-25 (Figure 3C). These results indicate that the SBD-2 peptides act as selective antagonists of the binding of synaphin to syntaxin and that synaphin and α-SNAP use distinct sites for binding to the SNARE complex.

To further evaluate the specificity of the SBD-2 peptide, we determined its protein binding partners. Detergent extracts of squid optic lobes were passed over an SBD-2 peptide affinity column, which retained only the SNARE proteins: syntaxin, SNAP-25, synaptobrevin, and a proteolytic fragment of synaptobrevin (Figure 3D). Using recombinant SNARE proteins, we found that syntaxin, but not SNAP-25 or synaptobrevin, bound directly to the SBD-2 column (data not shown). Thus, SNAP-25 and synaptobrevin are retained only indirectly, by binding to syntaxin. Different lines of evidence revealed that the binding of syntaxin to SBD-2 was specific. First, there was no detectable synaphin in the SBD-2 binding fraction (Figure 3E, lane 2), indicating that immobilized SBD-2 peptides compete with synaphin. Second, soluble SBD-2 peptide prevented syntaxin from binding to the SBD-2 column (Figure 3E, lane 3) while scrambled SBD-2 peptide had no effect (Figure 3E, lane 4). Again, α-SNAP did not prevent the binding of syntaxin to SBD-2 (Figure 3E, lane 5), consistent with the results shown in Figure 3C. These results indicate a high selectivity for SBD-2 and argue that the synaphin–syntaxin interaction is the only target of this peptide.

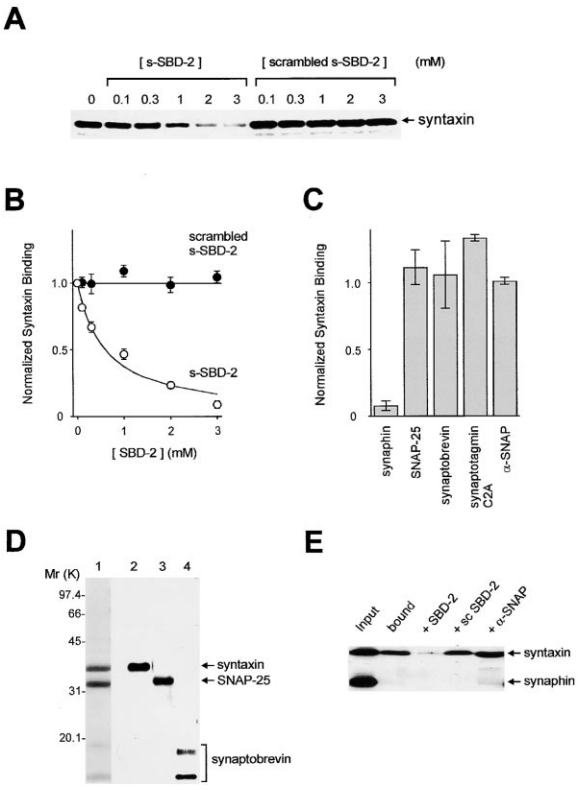

Figure 3. SBD-2 Peptides Compete with Synaphin but Not α-SNAP Binding to Syntaxin

(A) Increasing concentrations of the s-SBD-2 peptide prevented the interaction between GST-s-synaphin and syntaxin, while scrambled s-SBD-2 peptide had no effect.

(B) Dose dependence of s-SBD-2 inhibition. Points indicate means of four determinations and error bars indicate SEM.

(C) s-SBD-2 (3 mM) blocks the interaction of synaphin with syntaxin but not the interaction of syntaxin with other proteins. Data are normalized to binding measured in the absence of s-SBD-2 peptide and represent means ± SEM from three experiments.

(D) Binding of s-SBD-2 peptide to proteins in a detergent extract of squid optic lobe synaptosomes. s-SBD-2 was attached to beads and bound proteins were analyzed by SDS-PAGE and Coomassie blue stain (lane 1) or Western blot with anti-syntaxin antibody (lane 2), anti-SNAP-25 antibody (lane 3), or anti-synaptobrevin antibodies (lane 4).

(E) Binding specificity of SBD-2 peptide. The binding of both squid syntaxin and synaphin to SBD-2 beads was measured in buffer B (bound) or in the presence of soluble SBD-2 (5 mM), scrambled SBD-2 (5 mM), or His_6-α-SNAP (12 μM).

Synaphin Promotes Formation of SNARE Complex Oligomers

Because synaphin binds to the ternary SNARE complex (Figure 2A), it could cause a structural change in the SNARE complex. To consider this possibility, we exploited the fact that SNARE complexes are resistant to SDS at room temperature (Hayashi et al., 1994). Ternary SNARE complexes, purified from detergent extracts of squid synaptosomes via an SBD-2 affinity column, electrophoresed as a 60 kDa band characteristic of the SDS-resistant ternary SNARE complex (Figure 4A, left lane). Remarkably, recombinant synaphin caused the 60 kDa syntaxin-immunoreactive band to shift to molecular masses of 110 kDa and 130 kDa (Figure 4A, right lanes).

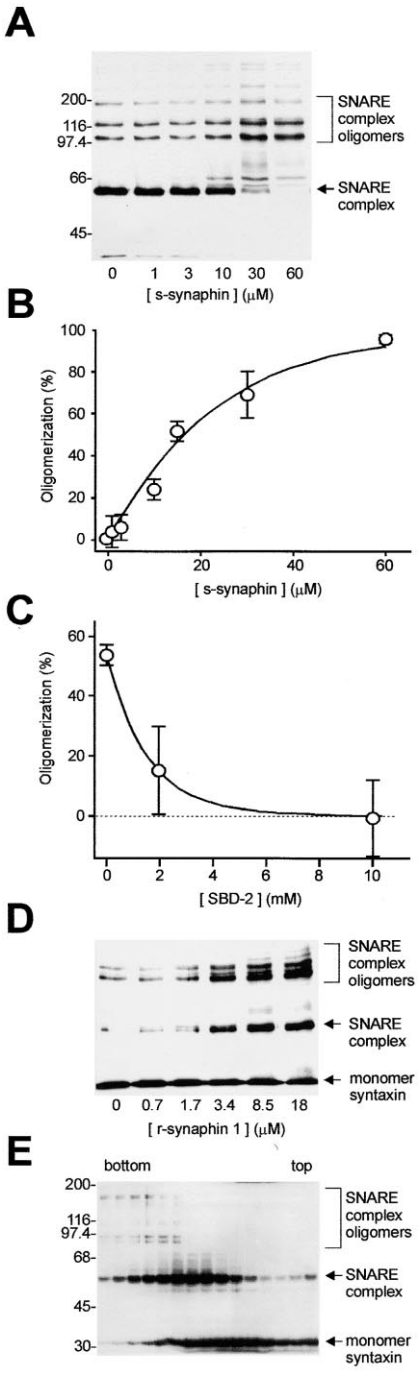

Figure 4. Synaphin Catalyzes the Oligomerization of SNARE Complexes

(A) Incubating detergent extract of squid optic lobe tissue with SBD-2 beads and varying concentrations of His_6-s-synaphin caused a shift in the distribution of syntaxin into higher molecular mass complexes. Syntaxin was detected via Western blotting.

(B) Relationship between concentration of His_6-s-synaphin and oligomerization of SNARE complexes, measured as a reduction in the amount of syntaxin found within 60 kDa SNARE complexes. Data represent means ± SEM from three experiments.

(C) SNARE complex oligomerization, induced by His_6-s-synaphin (15 μM), was inhibited by varying concentrations of SBD-2 peptide. Inhibition of oligomerization was measured as in (B).

(D) Oligomerization of ternary SNARE complexes formed by recombinant SNARE proteins. Addition of His_6-r-synaphin 1 caused a concentration-dependent increase in high-order oligomers and ternary SNARE complex, detected by Western blotting with an anti-syntaxin antibody.

(E) Analysis of SNARE complexes formed in the presence of His_6-r-synaphin 1 by glycerol density gradient centrifugation. Aliquots of each gradient fraction were subjected to SDS-PAGE and probed with anti-syntaxin antibody.

No detectable change in SNARE complex molecular mass was observed in the presence of 10 mM SBD-2 peptide (93.0 ± 2.4% SEM of control; n = 3) or the central region fragment of s-synaphin. Furthermore, addition of other charged proteins, such as cytochrome C, did not affect the 60 kDa SNARE complex. Very similar results were obtained with SNARE complexes solubilized from rat brain synaptosomes (data not shown). These data are consistent with synaphin inducing an oligomerization of SNARE complexes to form higher order structures.

To quantify this oligomerization activity of synaphin, we measured the disappearance of the 60 kDa SNARE band as a function of synaphin concentration (Figure 4B). Half-maximal oligomerization of SNARE complexes occurred with 15 μM synaphin; this is an upper estimate of the concentration required because the SBD-2 peptide used to purify SNARE complexes will compete with the synaphin. SNARE oligomerization was observed only when synaphin was freshly prepared; the activity of this protein apparently is sensitive to being frozen and thawed. Because SBD-2 prevents synaphin from binding to syntaxin (Figures 3A and 3B), this peptide should prevent synaphin-induced oligomerization of SNARE complexes. Indeed, the ability of synaphin to promote oligomerization of the SNARE complex was blocked by the SBD-2 peptide in a concentration-dependent manner (Figure 4C). Thus, binding to syntaxin is required for synaphin to oligomerize SNARE complexes.

We next determined whether oligomerization could be reconstituted with recombinant SNAREs. For this purpose, we incubated the cytoplasmic regions of SNARE proteins (0.3 μM each) with variable concentrations of r-synaphin 1. Similar to native SNAREs, the recombinant SNARE proteins also formed higher order oligomers in the presence of synaphin (Figure 4D). The effect was concentration dependent and half maximal at approximately 4 μM r-synaphin 1. However, in contrast to the decrease in 60 kDa ternary complexes observed with native SNAREs, synaphin increased the amount of ternary complexes formed by recombinant SNARE proteins. This difference apparently reflects the presence of monomeric SNAREs in the recombinant protein preparation. Collectively, these results show that synaphin-induced oligomerization does not require additional proteins or the membrane anchors of the SNARE proteins. Glycerol density gradient centrifugation confirmed that the higher molecular mass bands observed after synaphin treatment sedimented as expected for higher order SNARE complexes. Complexes generated by synaphin migrated faster than non-oligomerized ternary complexes (Figure 4E), corroborating that these oligomeric SNARE complexes are larger than the ternary SNARE complexes. Thus, the reduced mobility of the SNARE proteins observed on SDS-PAGE gels reflects the formation of genuine oligomeric complexes.

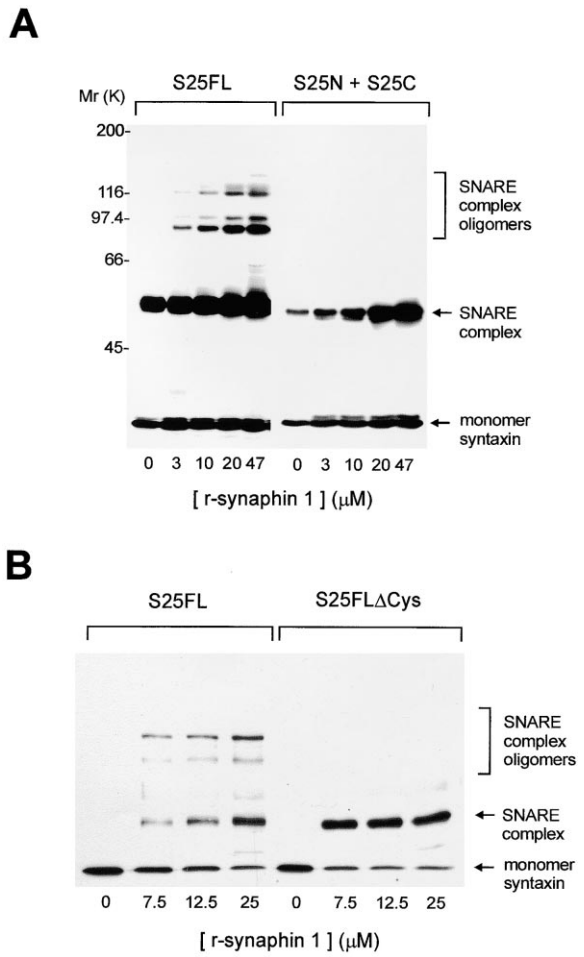

Figure 5. Synaphin-Induced Oligomerization of SNARE Complexes Requires an Intact Hinge Region

(A) While r-synaphin 1 oligomerized SNARE complexes containing the full-length SNAP-25 protein (S25FL), SNARE complexes formed by recombinant NH_2-terminal (S25N) and COOH-terminal (S25C) fragments of SNAP-25 did not oligomerize.

(B) Synaphin supports the oligomerization of SNAREs containing wild-type SNAP25 (S25FL) but not ΔCys-mutated SNAP-25 (S25FLΔCys).

We next performed experiments to determine how synaphin induces oligomerization of SNARE complexes. SNAP-25 is unique among SNAREs because it possesses two distinct coiled-coil domains that serve as binding sites for the other SNARE proteins (Chapman et al., 1994). This bivalent characteristic might allow SNAP-25 to cross-link SNARE complexes (Fasshauer et al., 1998; Poirier et al., 1998). We therefore examined whether SNAP-25 is important for the synaphin-induced oligomerization of SNARE complexes. First, we asked whether synaphin could cause oligomerization when the two coiled-coil domains of SNAP-25 were separated. For this purpose, we used separated NH_2-terminal (residues 1–95) and COOH-terminal (residues 125–206) fragments, instead of full-length SNAP-25, for the generation of SNARE complexes in the presence of r-synaphin 1. Confirming earlier observations of Poirier et al. (1998), ternary SNARE complexes were still formed by these SNAP-25 fragments (Figure 5A, right panel, lane 1). However, oligomers were not observed even when a large amount (47 μM) of r-synaphin 1 was added (Figure 5A, right panel, lanes 2–5). This contrasts with the behavior of full-length SNAP-25 (Figure 5A, left panel). Further, a mutant form of SNAP-25, in which four cysteines (residues 84, 85, 90, and 92) within the hinge region were replaced by alanines (ΔCys-mutated SNAP-25; Scales et al., 2000), was capable of forming ternary complexes when mixed with the other SNAREs (0.3 μM each; Figure 5B, right panel). But these mutant complexes also failed to oligomerize, even in the presence of a large excess of synaphin (Figure 5B, right panel). Under identical conditions, synaphin potently induced formation of oligomers from SNARE complexes containing wild-type SNAP-25 (Figure 5B, left panel). Thus, the central hinge region between the two coiled-coil domains of SNAP-25 is essential for synaphin-induced oligomerization of SNARE complexes.

SNARE Oligomerization Required for Neurotransmitter Release

To establish a physiological role for the binding of synaphin to the SNARE complex, we next microinjected SBD-2 peptides into the presynaptic terminal of the squid giant synapse. The peptides caused a potent inhibition of evoked transmitter release without affecting presynaptic resting or action potentials (Figure 6A). Both s-SBD-2 ($n = 11$) and r-SBD-2 ($n = 17$) were capable of completely inhibiting synaptic transmission. Blockade was concentration dependent, with half-maximal inhibition occurring at intracellular concentrations of approximately 1–2 mM as estimated by imaging of coinjected fluorescence dextran (Figure 6B). The similar potencies of both peptides in inhibiting transmitter release in vivo and in preventing binding in vitro argues that they act by preventing binding of synaphin to syntaxin. Synaptic transmission recovered over a time course of approximately 1–2 hr after peptide injection stopped (Figure 6C), presumably due to diffusion of peptide out of the presynaptic terminal. This indicated that inhibition was specifically due to the presence of peptide within the presynaptic terminal. Another indication of specificity is that peptides that had no effect on the binding of synaphin to syntaxin did not inhibit synaptic transmission (Figures 6D and 6E). For example, injection of scrambled s-SBD-2 had no measurable effect on synaptic transmission (decrease by 3.3 ± 1.9% SEM; $n = 4$), and the same was true for scrambled r-SBD-2 (4.4 ± 2.9% decrease; $n = 4$), s-SBD-1 (5.0 ± 9.9% decrease; $n = 4$), and carrier solution (3.2 ± 2.9% decrease; $n = 4$). For comparison, injecting similar amounts of s-SBD-2 inhibited synaptic transmission by 74.3 ± 6.2% ($n = 11$) and r-SBD-2 inhibited by 79.9 ± 4.8% ($n = 17$). Because of the parallel actions of these peptides on neurotransmitter release and on the interaction of synaphin with syntaxin, we conclude that the SBD-2 peptide acts by preventing this interaction in vivo. Thus, it appears that multimerization of SNARE complexes by synaphin is required for transmitter release.

Given the importance of calcium in triggering synaptic vesicle fusion, the action of the SBD-2 peptides could be due to changes in presynaptic calcium signaling. Imaging with a fluorescent calcium indicator dye

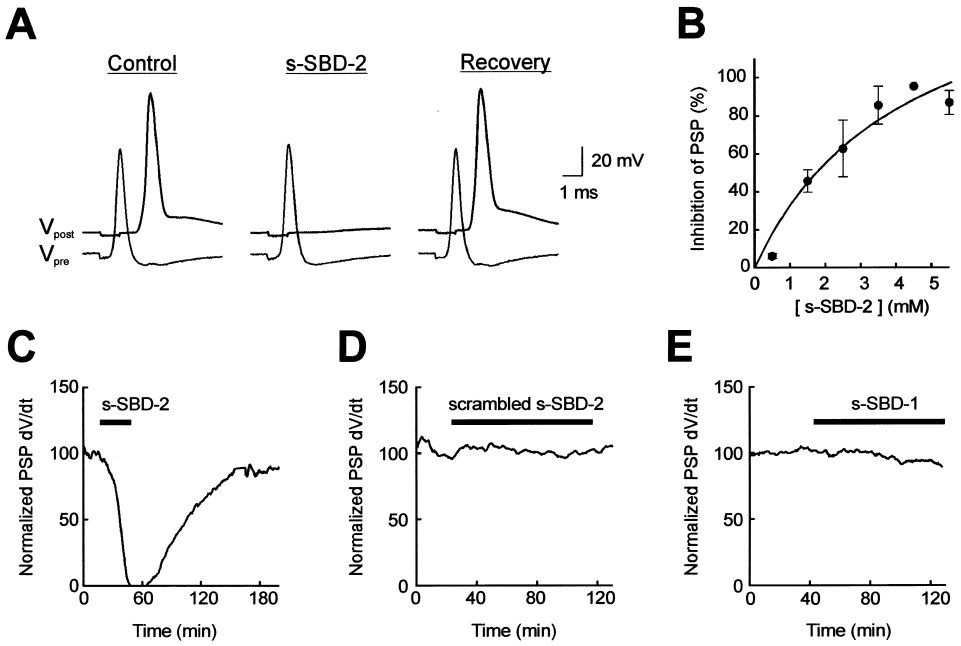

Figure 6. Inhibition of Evoked Transmitter Release by s-SBD-2

(A) Electrophysiological measurements of synaptic transmission before (left), during injection of s-SBD-2 (center), and 120 min (right) after stopping peptide injection. Note complete loss of postsynaptic potential.

(B) Relationship between estimated concentration of SBD-2 peptide within the presynaptic terminal and degree of inhibition of synaptic transmission, measured as the rate of rise of postsynaptic potentials (PSPs).

(C–E) Time-dependent changes in the rate of rise (dV/dt) of PSPs produced by s-SBD-2 (C), scrambled s-SBD-2 (D), and s-SBD-1 (E).

showed that microinjection of r-SBD-2 caused no significant change in calcium signals evoked by trains of presynaptic action potentials (Figure 7A). On average, calcium transients were 89 $\pm$ 5% of their peak control amplitudes following injection of r-SBD-2 and their time courses also were not detectably affected by peptide injection (Figure 7B). This indicates that the interaction of synaphin with syntaxin does not affect presynaptic calcium influx or removal.

A complete cycle of exocytosis and endocytosis of a synaptic vesicle requires at least 40–60 s (Betz and Bewick, 1992; Ryan et al., 1993). To determine when SBD-2 peptides work in this cycle, we delivered s-SBD-2 in a single, brief injection while rapidly stimulating the synapse (1 Hz) to measure the speed at which synaptic transmission was blocked (Figure 7C). Such injections decreased synaptic transmission by only a small amount, approximately 10%, as expected from the fact that a brief injection can deliver only enough SBD-2 to block a small fraction of the release sites within the large terminal (e.g., DeBello et al., 1995). This decline in synaptic transmission produced by the pulse of SBD-2 was described by an exponential function with an average time constant of 2.2 $\pm$ 0.5 s (n = 6). The rapid speed of inhibition indicates that the interaction of synaphin and syntaxin is essential for a reaction that lies temporally close to membrane fusion, rather than endocytosis.

To more directly examine the effects of SBD-2 on synaptic vesicle traffic, we examined the ultrastructure of presynaptic terminals injected with SBD-2. In these experiments, we injected r-SBD-2 until transmitter release was completely inhibited and the terminal was then fixed for electron microscopy. The general organization of these presynaptic terminals was unaltered by SBD-2 injection (Figure 7D). The spatial distribution of synaptic vesicles within the active zone also was little affected by SBD-2 injection (Figure 7E), other than a 32% increase ($p < .005$) in the number of docked vesicles, namely those vesicles whose centers were within 50 nm of the presynaptic plasma membrane (Figure 7F, first bar). This is consistent with a block of exocytosis at a prefusion step that follows vesicle docking. We therefore conclude that SNARE oligomerization induced by synaphin is required, within the last few seconds before vesicle fusion, in a reaction that follows synaptic vesicle docking and is independent of Ca channel gating.

Discussion

SNARE complexes can oligomerize into higher order structures (Hayashi et al., 1994; Pellegrini et al., 1995; Otto et al., 1997; Poirier et al., 1998), suggesting that assembly of SNARE complexes into a multimeric structure may occur prior to membrane fusion (Fasshauer et al., 1998; Poirier et al., 1998). We report here that synaphin promotes SNARE complex oligomerization, as shown by a synaphin-induced shift in the mobility of SNARE complexes on SDS gels and a parallel change in sedimentation behavior on glycerol density gradients (Figures 4 and 5). This action of synaphin was not observed by Pabst et al. (2000), perhaps because it requires fresh samples of recombinant synaphin and wild-

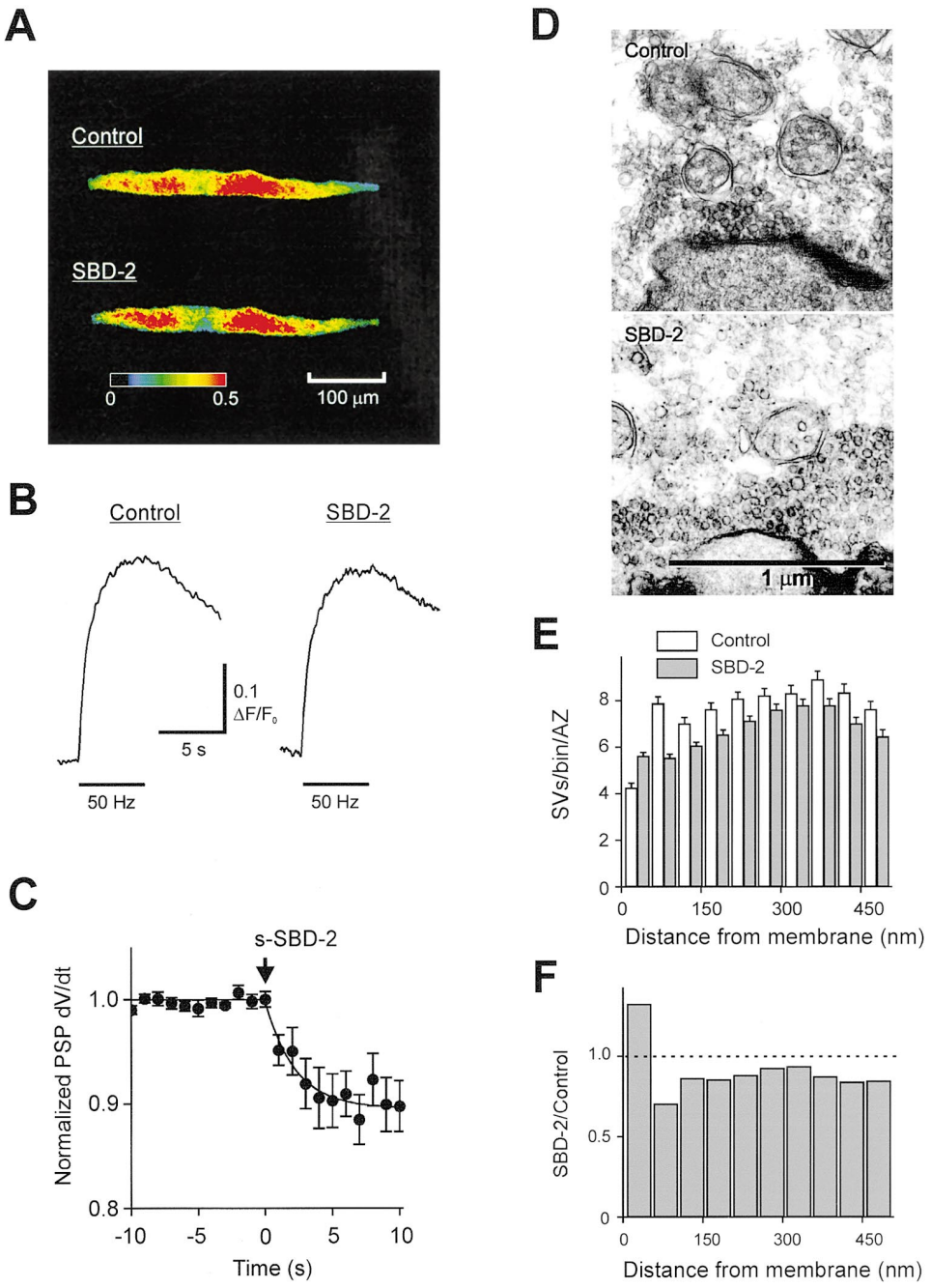

Figure 7. Mechanism of Synaptic Action of SBD-2

(A) Images of presynaptic calcium transients evoked by trains of presynaptic action potentials (50 Hz, 5 s) before (Control) and following (SBD-2) injection of r-SBD-2 peptide. Color scale indicates magnitude of stimulus-induced increase in dye fluorescence (ΔF), normalized by dividing by the initial fluorescence measured prior to stimulation (F_0).

(B) Time course of presynaptic calcium signals measured during trains of presynaptic action potentials (at bars) before (left) and during (right) injection of r-SBD-2.

(C) Rapid kinetics of inhibition of transmitter release upon injecting a single large volume of s-SBD2 (at arrow). Points indicate mean slope of postsynaptic potentials, normalized to their maximum value, measured in six experiments and curve indicates exponential function with time constant of 2.2 s.

(D) Electron micrographs of synaptic profiles from terminals injected with either r-SBD-2 (bottom) or scrambled r-SBD-2 (top).

(E) Distribution of synaptic vesicles (SVs) per active zone (AZ) at increasing distances from the presynaptic membrane. Two terminals were injected with r-SBD-2 (solid bars; n = 291 AZs), and another two were injected with scrambled r-SBD-2 (open bars; n = 218 AZs). Bars indicate means ± SEM.

(F) Relative densities of SVs in active zones of terminals injected with r-SBD-2 compared to those injected with scrambled r-SBD-2 (control).

type SNAP-25. Oligomerization of SNAREs appears to be important for synaptic vesicle fusion because the SBD-2 peptide, from the syntaxin binding domain of synaphin, blocks both oligomerization (Figure 4C) and neurotransmitter release (Figure 6B). Our studies thus support the idea that SNARE complex oligomerization is important for membrane fusion.

Our identification of the mechanism of action of synaphin in neurotransmitter release relied on the use of the SBD-2 peptide. Extensive in vitro analyses indicate that this peptide acts as a specific and competitive inhibitor of the interaction between syntaxin and synaphin. First, the peptide bound to syntaxin yet did not prevent binding of syntaxin to other proteins, most notably α-SNAP. The fact that the peptide did not prevent α-SNAP from binding makes the physiological actions of this reagent more interpretable than those of the central syntaxin binding domain construct or of full-length synaphin, which inhibit both synaphin and α-SNAP binding to syntaxin. Second, SBD-2 bound to syntaxin in a conformation-sensitive manner, having a much higher affinity for syntaxin within the 60 kDa ternary complex than for free syntaxin. Thus, even though the SBD-2 peptide displays a low affinity for binding to syntaxin (Figure 3B) and, consequently, inhibiting SNARE complex oligomerization (Figure 4C) and blocking neurotransmitter release (Figures 6A–6C), the peptide apparently acts with high specificity. We suspect that its low affinity results from the fact that the peptide is not structurally ordered, so that only a minor fraction assumes conformations suitable for binding to syntaxin.

Mechanism of Action of Synaphin

Our data showing that synaphin cannot oligomerize SNARE complexes containing the isolated NH_2- and COOH-terminal domains of SNAP-25 (Figure 5A) are consistent with synaphin causing oligomerization by cross-linking these complexes via the hinged, bivalent structure of SNAP-25. Further support for this idea comes from the observation that complexes containing SNAP-25 with ΔCys mutations in the hinge region also fail to oligomerize (Figure 5B). Because the NH_2-terminal fragment of SNAP-25 contains all four cysteine residues mutated in ΔCys SNAP-25, yet is unable to form oligomeric SNARE complexes, SNARE oligomerization is not simply due to the presence of these cysteine residues. Instead, it appears that oligomerization arises from the two coiled-coil domains of SNAP-25 residing in different SNARE complexes. Mutating the cysteines will change the secondary structure of the central region, which may prevent SNARE complex oligomerization by maintaining the two halves of SNAP-25 at an incorrect angle.

How do the two coiled-coil domains separate, given the conventional view that both halves of SNAP-25 contribute to formation of a single SNARE complex? We observed that synaphin enhances the formation of ternary complexes from recombinant SNARE proteins (Figure 4C), which was particularly obvious for the case of the ΔCys-mutated SNAP-25 that does not form oligomeric SNARE complexes (Figure 5B, right panel). Thus, synaphin may facilitate the assembly of free SNAREs into a "precomplex" with one end of SNAP-25 available for interaction with additional SNAREs (Figure 8A). Such precomplexes could correspond to the "loose" SNARE complexes that have been proposed to exist prior to calcium entry (Chen et al., 1999; Xu et al., 1999). We postulate that precomplex formation allows the free terminus of SNAP-25 to participate in a second SNARE complex and thus to cross-link complexes via the hinge region of SNAP-25. Depending upon the number of precomplexes involved, oligomers could form to include variable numbers of SNARE complexes (Figure 8A, lower right). We envision that formation of precomplexes involves binding of synaphin to binary complexes consisting of syntaxin and SNAP-25, as was observed in our experiments (Figure 2A). Precomplexes could form, albeit less efficiently, in the absence of synaphin, to account for observations that oligomers can be formed from mixtures of SNAREs alone (Poirier et al., 1998). This SNARE precomplex would become a stable ternary complex in the presence of SDS detergent (Figure 8A, lower left), accounting for the increased appearance of ternary complexes following treatment of recombinant SNAREs with synaphin (Figures 4C and 5B). In conclusion, synaphin seems to promote the formation of oligomers by both stimulating SNARE assembly and stabilizing an open conformation of SNAP-25.

Our data indicate that binding of synaphin to syntaxin plays an essential role in the synaptic vesicle fusion cascade. We presume that this is because synaphin promotes SNARE complex oligomerization, though synaphin may be important also because of its ability to form SNARE precomplexes (Figure 8A) or some other consequence of its association with syntaxin. Consistent with a previous proposal (Fasshauer et al., 1998; Poirier et al., 1998), we hypothesize that the oligomerization of SNARE complexes creates a three-dimensional ring structure that produces a scaffold required for calcium-dependent fusion of the synaptic vesicle with the presynaptic plasma membrane (Figure 8B, step 5). Such a structure would be analogous to the ring of hemagglutinin proteins that serves as a precursor for fusion of viruses with host membranes (Danieli et al., 1996; Bentz, 2000). In fact, electron microscopy suggests that purified SNARE complexes can form "ring" structures consisting of several SNARE complex rods (Hohl et al., 1998). In the case of viral fusion, the protein ring generates a microenvironment conducive to the final fusion of lipids in the two membranes (Chernomordik et al., 1999). Similar mechanisms may underlie regulated fusion during neurotransmitter release; for example, this ring could promote formation of a fusion pore between the vesicle and plasma membranes. However, cross-linked SNARE complexes are not absolutely required for membrane fusion. The yeast genome has no counterpart of synaphin (data not shown), complexes formed by yeast SNAREs are composed of four proteins that each contribute a single coiled-coil region (Fukuda et al., 2000), and yeast SNARE complexes apparently do not oligomerize. Given the slow kinetics of constitutive membrane fusion in yeast (e.g., Mayer et al., 1996), this evolutionary parallel between the presence of synaphin, SNAP-25-like t-SNAREs, and oligomerization of SNARE complexes suggests that a ring of SNARE complexes is only necessary for rapid, regulated exocytosis. This could also account for other reports that slow, SNARE-mediated fusion does not require the two coiled-coil

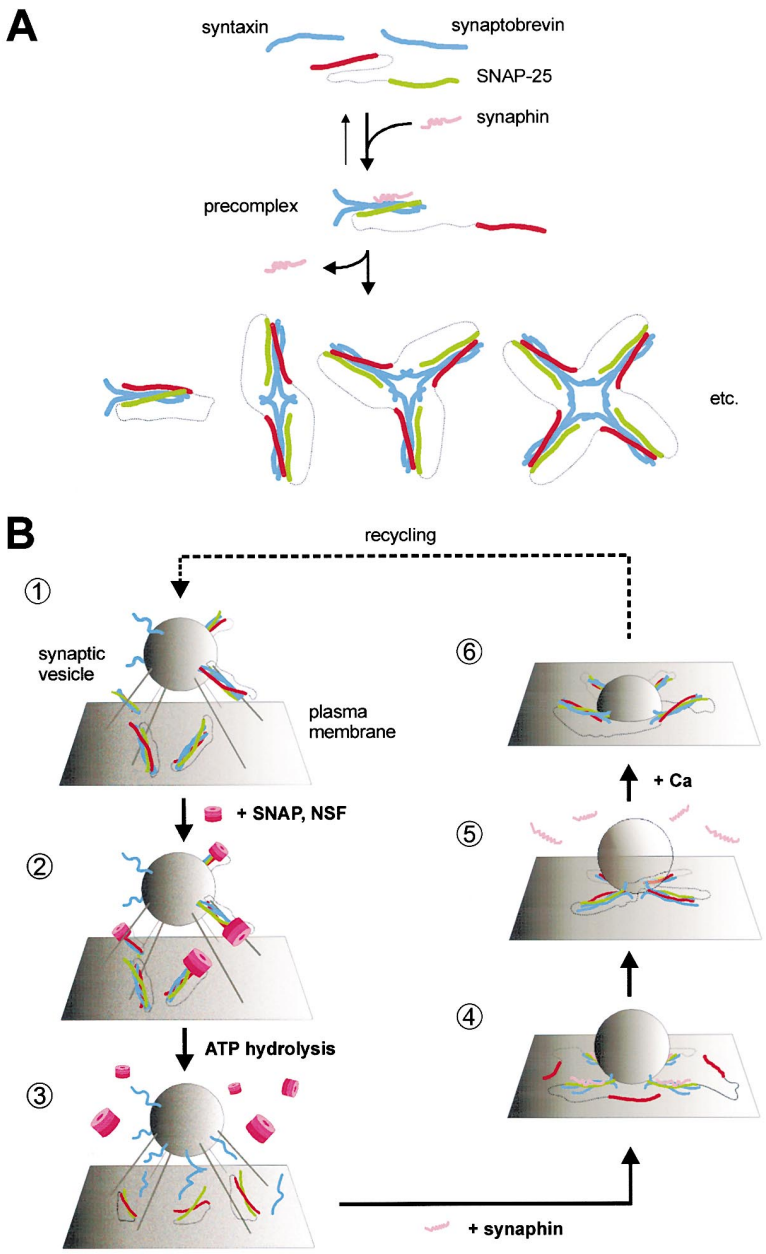

Figure 8. Mechanism of Synaphin Action

(A) Proposed reaction scheme for synaphin-induced oligomerization of SNARE complexes.

(B) A model for synaphin function during exocytosis. Participating proteins are depicted as indicated in the inset and numbers refer to steps described in the text.

domains of SNAP-25 to be connected to each other (Parlati et al., 1999; Scales et al., 2000). Perhaps a ring of SNARE proteins could form a structure necessary for synchronization of SNARE complex action, or for recruiting accessory proteins, such as synaptotagmin, that confer rapid calcium regulation (Südhof, 1995; Augustine et al., 1999). Consistent with these ideas, SBD-2 peptide (T. Blanpied et al., 1999, Soc. Neurosci., Abstract 25, 1749) or knockout of synaphin genes (Reim et al., 2001) impairs transmitter release evoked by presynaptic calcium influx but does not affect spontaneous transmitter release that does not require calcium influx.

Model for Synaphin Action in Synaptic Vesicle Trafficking

Synaphin competes with α-SNAP for binding to syntaxin in vitro (Figure 2B and McMahon et al., 1995), and α-SNAP promotes exocytosis (Chamberlain et al., 1995; DeBello et al., 1995). It has, therefore, been postulated that synaphin serves as a negative regulator of α-SNAP action, and the effects of microinjected r-synaphin 1 are compatible with this possibility (Ono et al., 1998). But while this hypothesis predicts that the SBD-2 peptides should either stimulate or have no effect upon transmitter release, we found complete inhibition by these peptides. Consequently, synaphin and α-SNAP each must have essential and separate functions in synaptic vesicle fusion.

SBD-2 peptide blocks synaptic vesicle exocytosis at a step after docking but before fusion. While there have been suggestions that SNARE proteins affect calcium channel gating (Bezprozvanny et al., 1995; Peters and Mayer, 1998; Wiser et al., 1999), it appears that synaphin does not serve such a function because the SBD-2 pep-

tide did not alter presynaptic calcium signals (Figures 7A and 7B). The actions of SBD-2 resemble the consequences of perturbing other SNARE complex components (Hunt et al., 1994; Broadie et al., 1995; DeBello et al., 1995; O'Connor et al., 1997; Schweizer et al., 1998) or synaptotagmin (Bommert et al., 1993), indicating that all of these proteins act late in the cascade of reactions that lead to synaptic vesicle fusion. Synaphin binds to syntaxin no later than 2 s before a synaptic vesicle fuses (Figure 7C), which is an upper estimate due to delays associated with peptide diffusion and the rate of vesicle turnover. The block produced by an α-SNAP peptide requires more than 4 s (DeBello et al., 1995), suggesting that synaphin may act later in the fusion cascade. Perhaps α-SNAP and its partner, NSF, act to dissociate *cis*-SNARE complexes on synaptic vesicles and/or the presynaptic plasma membrane (Mayer et al., 1996; Otto et al., 1997; Littleton et al., 1998; Robinson and Martin, 1998; Weber et al., 2000). These could include oligomerized SNAREs remaining from previous rounds of fusion (Hayashi et al., 1995; Pellegrini et al., 1995). Synaphin then would work after NSF and α-SNAP to help assemble and oligomerize *trans*-SNARE complexes between two membranes.

From the considerations presented above, we propose the following model for synaptic vesicle exocytosis (Figure 8B):

(1) The first step in exocytosis, docking of synaptic vesicles, does not require SNARE proteins because synaptic vesicles dock even when SNARE proteins are perturbed (Hunt et al., 1994; Broadie et al., 1995; DeBello et al., 1995; O'Connor et al., 1997; Littleton et al., 1998; Schweizer et al., 1998).

(2) Attachment of SNAP and NSF occurs at a priming step that follows vesicle docking and promotes fusion (DeBello et al., 1995; Banerjee et al., 1996; Schweizer et al., 1998).

(3) We presume that the function of NSF and SNAP is to separate *cis*-SNARE complexes on synaptic vesicles and, perhaps, on the plasma membrane. Because there is little synaphin in the synaptic vesicle fraction (Ishizuka et al., 1995), synaphin may not bind to *cis*-SNARE complexes in vivo.

(4) Synaphin works to promote the formation of SNARE precomplexes from free SNARE proteins on the two membranes, resulting in *trans*-SNARE complexes that bring the two membranes in close association (Hanson et al., 1997; Sutton et al., 1998).

(5) Subsequent oligomerization of the precomplexes generates a ring of SNARE proteins needed for efficient, calcium-regulated vesicle fusion. SBD-2 peptides prevent this step by inhibiting synaphin from promoting SNARE oligomerization.

(6) The resulting high-energy *trans*-SNARE complexes (Pellegrini et al., 1995) may produce membrane fusion (Weber et al., 1998; Chen et al., 1999) or serve as a late precursor to fusion (Coorssen et al., 1998; Ungermann et al., 1998), yielding release of neurotransmitter from the synaptic vesicle. The synaptic vesicle is then recycled to repeat this cycle of reactions.

In conclusion, our results indicate that synaphin facilitates the formation and subsequent oligomerization of SNARE complexes and that this function is essential for a late step in synaptic vesicle exocytosis. It is possible that synaphin or its homologs could be important for other rapid, SNARE-based forms of regulated membrane fusion as well. Our findings extend and revise earlier views of synaphin and suggest that synaptic vesicle exocytosis involves higher order SNARE structures at the site of vesicle-plasma membrane fusion.

Experimental Procedures

cDNA Cloning

Synaphin was purified from the cytosolic fraction of squid (*Loligo pealii*) optic lobes by immunoaffinity chromatography using 12C5 anti-rat synaphin 1 antibody (Ishizuka et al., 1997). The synaphin band was purified by SDS-PAGE, cleaved with cyanogen bromide, and HPLC-purified peptides were sequenced (Morita et al., 1992). Two partial sequences (MGGDEGE and DEGRVGR) were used to generate a 230 bp fragment by PCR with degenerate primers and optic lobe cDNA as a template. After verification by sequencing, this probe was used to screen a squid optic lobe λgt11 cDNA library and positive clones were sequenced. The entire coding region of the squid synaphin cDNA was ligated to pQE-30 (Qiagen) and pGEX-4T-1 (Pharmacia) to produce recombinant proteins. The GenBank accession number for the nucleotide sequence is AB003700.

Recombinant Proteins and Antibodies

The plasmid encoding ΔCys-mutated mouse SNAP-25 (cysteines 84, 85, 90, and 92 replaced by alanines) was a generous gift from Dr. R. Scheller (Howard Hughes Medical Institute, Stanford, CA). Plasmids for recombinant SNARE proteins were prepared by the method of Poirier et al. (1998). These fusion proteins were purified by glutathione-Sepharose and cleaved with thrombin to remove GST. A monoclonal antibody against rat syntaxin (6D2) was a gift of Dr. M. Takahashi (Mitsubishi Kasei Institute, Japan). Monoclonal antibodies against synaptobrevin (CL 10.1) and α/β-SNAP (CL 77.1) were gifts of Dr. R. Jahn (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany). Anti-SNAP-25 polyclonal antibodies were purchased from Alomone labs (Jerusalem).

Biochemical Procedures

Extracts from optic lobe synaptosomes (O'Connor et al., 1997) were prepared by solubilization in buffer A (150 mM NaCl and 20 mM Na-HEPES, pH 7.4) containing 2.5% (w/v) Triton X-100. Triton X-100 extract (10 μg of protein) was incubated with GST-s-synaphin (20–100 pmol) for 3 hr at 4°C with various concentrations of either peptides or His_6-α-SNAP. Bound proteins were affinity precipitated with glutathione-Sepharose beads and subjected to SDS-PAGE and Western blotting. Syntaxin was visualized by SuperSignal Substrate (Pierce) using 6D2 anti-syntaxin antibody (Yoshida et al., 1992), and quantified by comparing to recombinant His_6-s-syntaxin standards. Two other assays were performed to determine the effect of SBD-2 peptide on other protein–protein interactions. For immunoprecipitation, Triton X-100 extracts were incubated with His_6-s-synaptobrevin or His_6-α-SNAP in the presence or absence of 3 mM SBD-2 peptide and bound proteins were precipitated with 6D2 anti-syntaxin antibodies. Alternatively, His_6-s-SNAP-25 or His_6-s-syntaxin were incubated with GST-s-syntaxin or GST-s-synaptotagmin C2A, respectively, in the presence or absence of SBD-2 peptide and bound proteins were affinity precipitated with glutathione-Sepharose beads. To assay syntaxin binding to the C2A domain of synaptotagmin, 1 mM $CaCl_2$ was added to buffer B (buffer A containing 0.1% (w/v) Triton X-100). SulfoLink Coupling Gel (Pierce) was used for immobilization of s-SBD-2 peptide with an additional NH_2-terminal cysteine according to the manufacturer's instructions (1.0 mg peptides/ml gel). Triton X-100 extract (100 mg of protein) was applied to s-SBD-2 beads (2 ml) and bound proteins were analyzed by 12% SDS-PAGE and Coomassie blue staining with GelCode Blue Stain (Pierce). Oligomerization of ternary SNARE complexes was measured by affinity-purifying these complexes on SBD-2 beads and then incubating with His_6-synaphins for 2–16 hr at 4°C. Each sample was directly diluted in SDS sample buffer and incubated at 37°C (unboiled) for 5 min (Pellegrini et al., 1995), subjected to 7.5% SDS-PAGE, and Western blotted. High molecular mass complexes

were probed with 6D2 anti-syntaxin antibodies. The efficiency of oligomerization was determined as percent reduction of syntaxin immunoreactivity in ternary (60 kDa) SNARE complexes. Glycerol density gradient centrifugation was done by incubating a mixture of the three recombinant SNAREs (0.1 μM each), prepared as described above, with or without 50 μM His_6-r-synaphin 1 at 4°C for 16 hr. The reaction mixture was fractionated on 10%–25% (w/v) linear glycerol gradient. Each fraction was immediately incubated with SDS sample buffer and subsequently analyzed by immunoblotting with 6D2 anti-syntaxin antibodies.

Physiological Measurements

Transmission at giant synapses of squid stellate ganglia was examined using electrophysiological techniques (e.g., Bommert et al., 1993). In brief, one presynaptic microelectrode containing 3 M KCl was inserted into the presynaptic axon to inject depolarizing current that evoked action potentials, while a second was used to microinject reagents and record the presynaptic action potential. A third microelectrode containing 3 M KCl was inserted into the postsynaptic cell to monitor postsynaptic responses. Peptides were synthesized by L. Bonewald (University of Texas, San Antonio) or Sigma Genosis (The Woodlands, TX) with alkylated NH_2 termini and amidated COOH termini. For microinjection experiments, peptides (s-SBD-2, r-SBD-2, scrambled s-SBD-2 = ELRYDRITKMKGEQK, and scrambled r-SBD-2 = RYKQIKEVKQKRLGD) were dissolved in carrier solution (200 mM KCl, 100 mM taurine, 200 mM K-isethionate, 50 mM K-HEPES, pH 7.4). Fluorescence imaging procedures are described in Smith et al. (1993). Presynaptic terminals were injected with Calcium Orange (Molecular Probes) and dye fluorescence was imaged with an intensified CCD camera (Photon Technology International, Inc., NJ). Images were stored at 30 Hz on an optical disc recorder and analyzed offline with Image-1 software (Universal Imaging, Philadelphia, PA). Dye fluorescence was measured within defined presynaptic areas during action potential trains; calcium-induced increases in dye fluorescence were normalized, by dividing by the resting fluorescence of the areas, to yield a signal that is proportional to the change in presynaptic calcium concentration (Hunt et al., 1994).

Electron Microscopy

Terminals injected with SBD-2 peptide or scrambled SBD-2 peptide were fixed and processed as described in Sanchez et al. (1990). EM images were digitized and analyzed (using Image-1 software) as described previously (Burns et al., 1998).

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